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(54) Title: PRIMATE ERYTHROCYTE BOUND MONOCLONAL ANTIBODY HETEROPOLYMERS

(57) Abstract

The application relates to the use of antibodies in therapy. Monoclonal antibody heteropolymers are described which comprise a monoclonal antibody specific for the CR1 receptor on primate red blood cells covalently linked to a second monoclonal antibody specific for an antigen to be cleared from the circulatory system. The monoclonal antibody heteropolymers may be injected directly into a patient's circulatory system. Alternatively, red blood cells removed from a patient or a suitable donor may be contacted with the monoclonal antibody heteropolymers and then administered to the patient.

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Description

Primate Erythrocyte Bound Monoclonal Antibody Heteropolymers

Technical Field

This invention is directed to mammalian primate erythrocytes to which have been bound cross-linked monoclonal antibodies (heteropolymers) specific for both the erythrocyte complement receptor protein (CR1), and (a 2nd antibody bound thereto specific for) a circulating antigen. Methods of using these "franked" erythrocytes in diagnostic or assay methodology and therapeutic applications are also addressed.

Background Art

Mammalian primate erythrocytes (RBC's) have been identified as essential to the body's ability to clear antibody/antigen immune complexes from the blood. Specifically, the RBC receptor (CR1), known to be specific for certain activated complement proteins (C3b, C3bi and C4b), has been implicated as playing an important role in the primate's defense against microorganism infection by facilitating the neutralization and clearance of certain pathogenic substances. Other evidence shows that the binding of these immune complexes to RBC's at the CR1 site provides a vehicle for rapid clearance of potentially pathogenic immune complexes from circulation. Enhancement of phagocytosis and circulatory transport of immune complexes have both been advanced as mechanisms by which the RBC's function in this immune response have been described. See, e.g., <u>Nelson</u>, <u>Science</u> 118, 733-737 (1953) and <u>Hebert et al</u>, <u>Kidney Int.</u>, 31, 877-885 (1987). In any event, defects in aspects of this RBC clearance

method have been demonstrated to be at least statistically related to a number of diseases and are believed to presage various disease activities.

Notwithstanding the importance of this function of the RBC and the immune system, it is apparent that the RBC binding and clearance capacity therefore is confined to immune complexes recognized by the CR1 receptor, that is the immune complexes must contain large amounts of at least one of the activated Complement proteins C3b, C3bi, or C4b. Thus, the mammalian primate or human body has no normal capacity to take advantage of the clearance system provided by the RBC binding ability to remove antigens not complexed with the identified activated complement proteins. It remains an object of those of skill in the art to augment the natural capacity of the mammalian circulatory system to clear antigens through RBC binding ability to include the ability to bind immune complexes (antigen/antibody complexes) *via* CR1 to RBC's in the *absence* of activated complement proteins. These augmented RBC's would be useful both in a therapeutic sense, as well as in an assay mode to identify the presence or absence of specific antigens.

Disclosure of the Invention

Specific monoclonal antibody heteropolymers are prepared from Mabs specific to the CR1 RBC receptor and Mabs to at least one other antigen and are then heteropolymerized using established techniques, as reported in U.S. Patent Application Serial No. 07/592,801 filed October 4, 1990. This heteropolymer binds readily to RBC's in whole blood, in numbers in good agreement with the number of CR1 sites available. The RBC's, if franked *in vivo* with the heteropolymer, will then bind

the antigen for which the remaining Mab is specific. These RBC's then can act therapeutically by facilitating the neutralization and clearance from the circulation of the bound antigen.

Alternatively, if introduced into a blood sample, franked RBC's bind quite rapidly to the antigen for which the second Mab is specific, and can also be used to assay for the presence of that particular antigen. If necessary, labeling of the heteropolymer and/or the antigen with, e.g., radioactive iodine, can facilitate bound RBC counts, and both qualitative and quantitative assessment of the antigen presence. Specifically, the heteropolymer-franked RBC's can be used in clinical assays for antigens in the circulation as demonstrated by the following example: Franked RBC's would be added to the plasma or anti-coagulated blood and allowed to bind the putative target antigen. After a wash the presence of the antigen bound to the RBC's would be revealed using appropriately labelled (e.g. either with ^{125}I, or enzyme-linked) second antibodies to the target antigen. Such assays can be either qualitative or quantitative.

RBC's removed and isolated may also be used as therapeutic agents. Once franked with heteropolymer, these RBC's can be reintroduced into the patient, where, in the bloodstream, free antigen will be bound and immobilized on the RBC, and cleared in accordance with the body's RBC clearing mechanism, which has been identified but is not completely understood. The franked RBC's can be specific for known antigens, such as HIV (the AIDS virus), or for substances which, if present in large amounts, can induce or aggravate disease states, such as low-density lipoproteins, or cause adverse biological effects, such as elevated hormone levels.

Given the general ease with which Mabs can be prepared for any known antigen, the variety of franked RBC's imaginable is unlimited. Any antigen or immunogen found in the bloodstream can be addressed by this therapeutic method.

In an alternative embodiment, RBCs are franked with a "cocktail" of several heteropolymers which, in addition to binding the target antigen, also bind to several distinct and non-overlapping sites on CR1 of the RBC. These points are identified, by e.g., Mabs 1B4, HB8592, and 57F. Experiments have demonstrated that by using two or more non-overlapping Mabs for binding to CR1 on the RBC, the number of Mab heteropolymers that can be bound to a single RBC is increased in numbers in good agreement with the number of available binding sites. This augments the capability of a relatively small number of RBC's to bind to a relatively larger amount of antigen, and can further facilitate removal of the antigen through the normal immune clearance system.

Brief Description of the Drawings

Figure 1 illustrates heteropolymer mediated binding of Human IgG to erythrocytes as compared to controls (unfilled columns in Fig. 1b). Figure 2 reflects heteropolymer mediated saturation of binding of DNP_5BGG to human erythrocytes.

Figures 3 A and B reflect in vivo clearance of injected antigen pursuant to the claimed invention.

Figures 4A and B gives similar data for another test individual.

Figure 5A gives similar clearance data for an independent test in another individual.

Figure 5B traces the degree of change in ^{51}Cr label throughout a clearance test.

Figures 6A, 6B and 6C reflect clearance of heteropolymers of the claimed invention from the circulation of a Rhesus monkey.

Best Mode for Carrying Out the Invention

As noted above, the flexibility of the franked RBC's of this invention in addressing a variety of disease states is limited only by the varieties of different antigens present in or accessible to the circulatory system and to which Mab can be prepared. A variety of Mab heteropolymers have been prepared. In order to attach to the RBC, the antigen-specific Mab is cross-linked with a Mab to the RBC complement receptor, CR1. Methods of cross-linking these antibodies are known to those of skill in the art. In the examples set forth below, some cross-linked heteropolymers were prepared using N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) according to established, published procedures. For details as to this procedure, see, e.g., Karpovsky et al, J. Exp. Med. 160, 1686-1701 (1984); Perez et al, Nature, 316, 354-356 (1985) or Titus et al, Journal of Immunology, 139, 3153-3158 (1987). In an alternative embodiment, heteropolymers are formed by biotinylating a Mab, incubating the biotin-bearing Mab with an avidin or strepavidin molecule, and then introducing a second Mab, also bearing a biotin linker arm. The thus-formed heteropolymer is a Mab-biotin-avidin/strepavidin-biotin-Mab sandwich. Other procedures are known to those of

ordinary skill in the art. A full listing of Mab heteropolymers prepared appears in Table 1, infra.. Prototype antigens selected for targeting through preparation of appropriate heteropolymers include dinitrophenylated bovine gamma globulin ($DNP_{55}BGG$), and human IgG. Both antigens and heteropolymers were iodinated by the IODOGEN method (Fraker et al, Biochem. Biophys. Res. Commun. 80, 849-857 (1978). Iodination provides one protocol for assay utilization, but of course need not be practiced for the therapeutic aspects of the claimed invention.

Assays for CR1 levels on isolated RBC's followed standard methods, revealing about 200-500 epitopes per RBC, as bound to by anti-CR1 Mabs 1B4, 3D9 and HB8592.

Details regarding RBC binding, binding kinetics and observed values follow below.

Examples:

1) Direct sensitization and binding isotherm analyses: Between 0.1 and 1.0 ml of a 10%-50% dispersion of washed RBC's in bovine serum albumin/phosphate buffered saline (BSA-PBS) were reacted for 1 hr at room temperature, with shaking, with varying amounts (10-50 ul) of a dilution of one or more of the heteropolymers. The RBC's were then washed 3 times in BSA-PBS (to remove excess unbound heteropolymer) and, after reconstitution in either BSA-PBS or normal human serum (for experiments with ^{125}I-human IgG and $DNP_{55}BGG$, respectively), mixed with a small volume of ^{125}I-probe. After a further incubation (usually 1 hr at room temperature, with shaking), RBC-bound and free ^{125}I-antigens were separated by either of two procedures: RBC's were spun through oil

(typically 150 ul of reaction mixture was layered on 200 ul of a dibutyl-dinonyl phthalate mixture), or simply processed by two cycles of centrifugation and washing in BSA-PBS. RBC-associated ^{125}I counts were quantitated in a Beckman 5500 gamma counter.

2) "Whole Blood" binding kinetics: a) In most procedures blood was drawn into Alsever's and centrifuged. A portion of the supernatant was removed, and after the blood cells were redispersed to a final hematocrit of 50%, a small amount of ^{125}I-$DNP_{55}BGG$ antigen was added. Varying amounts of heteropolymer were added directly to aliquots of these "whole blood" dispersions containing ^{125}I-DNP_{55},BGG, and incubated with shaking at 37°C. RBC associated ^{125}I counts were determined at varying time points after centrifugation and washing steps. Selected aliquots of the reaction mixtures were also centrifuged through percoll to confirm that only RBC's (not white cells) bound the ^{125}I-antigen. In some of these "whole blood" experiments, instead of using Alsever's as an anti-coagulant, blood was drawn into EDTA or citrate and used at once in a similar manner. A few comparable "whole blood" experiments were also performed with ^{125}I-human IgG as the target antigen. In these experiments washed RBC's were dispersed in BSA-PBS, to avoid the potential confounding effect of endogenous serum-associated IgG. b) In other kinetic experiments one volume of RBC's was franked with saturating amounts of the heteropolymer, and after three washes was added to 10 volumes of anti-coagulated blood containing ^{125}I-$DNP_{55}BGG$, and incubated at 37°C. Aliquots of the dispersions were processed periodically to determine RBC-associated ^{125}I counts.

Direct binding of ^{125}I-heteropolymers to a number of

matrices was determined in procedures analogous to those described above. For example, duplicate aliquots of 100 ul of ^{125}I-heteropolymer #4 (see below) were incubated for one hour at room temperature, with shaking, with either 100 ul of a 50% dispersion of human RBC's, or 100 ul of a 33% dispersion of human IgG-Sepharose. Samples were then subjected to two cycles of centrifugation and washing and the levels of matrix-bound ^{125}I counts were determined. Direct binding to human RBC's of the ^{125}I-heteropolymers was also determined as a function of time at 37°C.

Control experiments tested for the specificity of antigen binding by heteropolymer treated RBC's and verified the requirement for CR1. These experiments included the use of heteropolymer-treated sheep RBC's (which lack CR1), naive (untreated) human RBC's, and excess homologous monomeric Mabs (in ascites fluid) which blocked the action of the heteropolymers.

Results

<u>Preparation and Initial Characterizations of Heteropolymers</u>. We prepared a number of heteropolymers by SPDP cross-linking, and examined the ability of these heteropolymers to react with human RBC's and facilitate binding of specific antigens. Preliminary data (Table 1), using mixtures of saturating amounts of <u>unfractionated</u> material (containing heteropolymers and non-cross-linked monomers), demonstrated specific RBC-associated binding of the ^{125}I-antigens. An excess of ^{125}I-antigen was used in order to determine the <u>maximum</u> number of ligands bound per RBC. For each heteropolymer mixture the results (Table 1) are in good agreement with the typical number of CR1 epitopes (200-500) recognized by the anti-CR1 Mabs.

Heteropolymer mixture #1 can facilitate binding _via_ two _noncompeting_ Mabs to CR1, 1B4 and HB8592. This mixture can, therefore, place approximately twice as many anti-IgG heteropolymers on the RBC's as a heteropolymer containing only one anti-CR1 Mab. The maximum ^{125}I-human IgG bound to such "doubly-franked" RBC's is nearly equal to the sum of the ^{125}I-IgG bound to RBC's franked with two individual components of the mixture (Table 1); this illustrates _the principle of additivity_. Dose-response experiments with heteropolymer #1 and other heteropolymers (Table 1, and see below) confirm that RBC binding of both heteropolymer and ^{125}I-antigen is saturable. "Background" binding of antigen to naive RBC's is low, and use of heteropolymers with "irrelevant" specificities for the target ligands (e.g. 8E11 (anti-C3b) X HB8592) gave no binding (Table 1).

Binding Isotherms with Isolated Heteropolymers. Heteropolymer mixtures were further purified by gel permeation chromatography, and the highest mw subfractions (ca. corresponding to trimers and larger species) were used to quantitate binding (Figures 1 and 2). In these experiments binding of ^{125}I-antigens to franked RBC's was determined, after two cycles of centrifugation and washing with BSA-PBS, by direct counting of the RBC pellets.

At saturating input of heteropolymer, the maximum number of antigen molecules bound per RBC is in good quantitative agreement with our initial survey results using unfractionated heteropolymer mixtures and centrifugation through oil to separate bound from free ^{125}I-antigen. These experiments confirm that binding is saturable, since use of excess quantities of a single heteropolymer or ^{125}I-antigen does not increase binding

beyond the saturation level (typically 200-1000 antigens per RBC, Figures 1 and 2). Analysis of results with blood from two donors (Figure 1a and 1b) demonstrates that maximum binding reflects the number of CR1 epitopes per RBC characteristic of the individual donor. The principle of additivity is also illustrated in experiments in which RBC's were franked with a combined mixture of two heteropolymers (Figure 1a). The combined action of the two heteropolymers in facilitating binding of the ^{125}I-antigen is close to the sum of the action of each species individually.

Bi-specificity of the heteropolymers was demonstrated in inhibition experiments using an excess of homologous monomeric Mab. Our goal was either to block binding of heteropolymer to RBC's (using an appropriate anti-CR1 Mab to thus preclude binding of ^{125}I-$DNP_{55}BGG$), or to inhibit directly binding of ^{125}I-$DNP_{55}BGG$ to franked RBC's (using the appropriate monomeric anti-DNP Mab). In all cases more than 90% of specific binding was reduced by these procedures (Figure 1b). Sheep RBC's lack CR1, and as anticipated, heteropolymers directed against CR1 do not facilitate binding of the ^{125}I-antigen to sheep RBC's (Figure 1b). Finally, the dual specificities of two of the heteropolymer mixtures was confirmed by labelling them with ^{125}I and examining their binding to human RBC's and to a Sepharose 4B matrix containing their respective target antigens (Table 2). The results demonstrate that the isolated polymers bind to both of their respective matrices, and also confirm that their direct binding to human RBC's is rapid at 37°C.

TABLE 1

Survey of Cross-Linked Mab Heteropolymer Mixtures in facilitating Antigen Binding to RBC's

A. Binding of ^{125}I-Human IgG

Hetero Polymer #	Mab #1 (Specificity) X Mab #2 (Specificity) *	Molecules** IGG Bound/ RBC
1	1B4(anti-CR1) X HB43 (anti-IgG) and HB8592 (anti-CR1)	1150
2	HB43(anti-IgG) X HB8592 (anti-CR1)	601
3	HB43(anti-IgG) X 1B4(anti-CRI)	492
4	HB43(anti-IgG) X HB8592(anti-CR1)	357
5	HB43(anti-IgG) X 1B4(anti-CR1)	479
6	HB43(anti-IgG) X 3D9(anti-CR1)	355
7	HB43(anti-IgG) X 57F(anti-CR1)	387
Control	8E11(anti-C3b) X HB8592(anti-CR1)	-2

B. Binding of $^{125}I-(DNP)_{55}BGG$

DNP$_{55}$BGG Heteropolymer	Mab #1 (Specificity) X Mab #2 (Specificity) *	Molecules Bound/ RBC
8	3D9(anti-CR1) X 2A1(anti-DNP)	191
9	3D9(anti-CR1) X 23D(anti-CR1)	243
10	23D1(anti-DNP) X HB8592(anti-CR1)	129
11	23D1(anti-DNP) X 1B4(anti-CR1)	255
12	23D1(anti-DNP) X 3D9(anti-CR1)	196
13	HB8592(anti-CR) X 2A1(anti-DNP)	95
14	HB8592(anti-CR1) X 23D1(anti-DNP)	133
15	1B4(anti-CR1) X 23D1(anti-DNP)	279
16	1B4(anti-CR1) X 2A1(anti-DNP)	236
Control	8E11(anti-C3b) x HB8592(anti-CR1)	-11

C. Demonstration of Saturation of Binding with Heteropolymer #1 and ^{125}I-IgG

Relative Heteropolymer Concentration	Relative ^{125}I-Human IgG Concentration	Molecules** IgG Bound RBC
5	5	994
5	1	868
5	0.2	343
1 ***	1 ****	777
0.2	1	205

Table 1 footnotes

* For each heteropolymer listed, the first Mab was reduced with dithiothreitol (after reacting with SPDP) and then coupled to the second SPDP-reacted Mab. Heteropolymers #2 and #4 represent preparations with HB8592 purified via protein G and octanoic acid-50% saturated ammonium sulfate, respectively. Heteropolymer # 1 was prepared by simultaneously reacting a cocktail of SPDP-coupled and reduced 1B4 and HB8592 with SPDP-coupled HB43.

** Binding was determined by centrifuging RBC's through oil. Background binding to naive human RBC's was 40 human IgG and 60 $DNP_{55}BGG$ per RBC respectively, and was subtracted to give the net specific binding reported. In parts A and B predetermined saturating inputs of both heteropolymer and ^{125}I-antigen were used.

*** "1" corresponds to 3.0 ug/ml of heteropolymer in a 12.5% hematocrit.

**** "1" corresponds to 0.92 ug/ml ^{125}I-human IgG in a 12.5% hematocrit.

TABLE 2

Binding of ^{125}I-Labelled Heteropolymers to Human RBC's or to Sepharose Coupled Ligands

	% Bound *	
	Human RBC's	IgG (or DNP)-Sepharose
#4, unfractionated mixture (HB43(anti-IgG) X HB8592(anti-CR1))	45 ± 5	55 ± 5
#4, isolated polymer fraction	65 ± 3	86 ± 5
#11, unfractionated mixture (23D1(anti-DNP) X 1B4 (anti-CR1))	34 ± 6 **	86 ± 5
#11, isolated polymer fraction	72 ± 2 ***	85 ± 5

Table 2 footnotes

* Bound after incubation (with an excess of binding matrix) for one hour at either room temperature (Sepharose samples) and/or 37 °C (RBC's were examined at both temperatures). IgG Sepharose was used as the binding matrix for heteropolymer #4, and DNP-Sepharose (containing a dinitrophenylated Mab to IgM) was used for #11. All samples were corrected for background binding (5% or less) to sheep RBC's or naive (unreacted) Sepharose.

** Binding was 30% and 32% respectively after incubation for either two or five minutes at 37°C.

*** Binding was 57% and 69%, respectively, after incubation for either two minutes or five minutes at 37°C.

PRIMATE STUDIES:

Materials and Methods

Mab and Heteropolymer Preparation. Purified Mab to CR1, the DNP group, and human IgG were biotinylated following published procedures Wilchek et al, Meth. Enzym., 184, Avidin-Biotin Technology (1990) using the "long-arm" biotinylating agent biotinyl-N-hydroxy succinimide (Vector Laboratories, Burlingame, CA) at molar inputs of biotin to Mab between 5 to 1 and 20 to 1. The Iodogen method Fraker et al, Bioc & Biop. Review, 80:849 (1978) was used to label both naive and biotinylated Mab with ^{125}I. DNP_5BGG Little et al, Meth. Immun. Immunochem, 1:128 (1967), was similarly labelled with either ^{125}I or ^{131}I. Preparation of soluble cross-linked heteropolymer (HP) which specifically bound to primate E was accomplished by first incubating (at 37°C for 30 min) a biotinylated Mab to CR1 (either $1B4_{10}$ or $E11_{10}$) with excess strepavidin (SA) (Gibco BRI, Gaithersburg, MD). Subsequently a biotinylated Mab to the DNP group ($23D1_5$) was incubated with this complex, and the resultant HP was then used without further purification. Detailed dose response tests were performed to determine the optimum inputs of biotinylated first Mab (anti-CR1), SA, and biotinylated second Mab (antiDNP) in the generation of soluble HP which bound to human and other primate erythrocyte (E) and facilitated antigen (Ag) binding. In all cases we verified that the HP used in these studies neither directly bound to, nor facilitated Ag binding to sheep E, which lack CR1 (22).

RIA. Varying inputs of radiolabelled HP (either the anti-CR1 Mab or anti-DNP Mab portion of the HP was radiolabelled) were incubated at 37°C for 5-15 min with a

50% dispersion (in homologous serum) of human or primate E [a "whole blood" simulation Ross et al, J. Immunol., 135:2005 (1985)]. Binding was determined by counting the E pellets after centrifugation and washing. To quantitate HP-mediated binding of Ag (DNP_5BGG) to E, the radiolabelled Ag was first added to the "whole blood" dispersion, and after varying amounts of HP were added, the samples were processed similarly. In some in vitro Ag-binding experiments freshly drawn anti-coagulated blood was used, and Ag binding via the HP was also demonstrable.

The number of CR1 epitopes per E was determined by incubating an excess of ^{125}I-labelled Mab to CR1 with isolated and washed E followed by one of two procedures for separation of E-"bound" from "free" Mab: 1) the incubation mixtures were layered on dibutyldinonyl phthalate mixtures, and after a centrifugation step the samples were frozen and the E pellets were cut off or counted; 2) alteratively, the E dispersions were simply pelleted, and after washing, the E pellets were counted. In all RIA, sheep E controls were included to provide a background correction, which was less than 10% of specific binding to human E.

In Vivo Experiments. We followed protocols similar to those we previously reported for studying clearance of IC from the circulation of both non-primates and primates. See, e.g., Taylor et al, J. Immunol. 139:1240 (1987). The doses of Ag and HP chosen in these experiments were optimized based on titration experiments performed in anti-coagulated whole blood samples from both humans and monkeys: 1 ml of radiolabelled DNP_5BGG (3-25 μg) in PBS was infused within 30 seconds into the catheterized saphenous vein of a 1 kg squirrel monkey

sedated with ketamine and maintained under anesthesia with halothane. After 20-35 min, 1 ml of HP (0.25-1.0 mg, either radiolabelled or unlabelled) was injected. Multiple blood aliquots (anti-coagulated in citrate) were collected over the entire course of the experiment to determine both the "endogenous" clearance rate of the Ag, and the effect of the HP on its clearance and E binding. Within 10 min of collection each blood sample was centrifuged and the cell pellet washed and counted to determine binding. The supernatants were combined and counted, and aliquots were examined to determine net 5% TCA-precipitable counts. Initially after injection, ^{125}I and ^{131}I counts recovered in the plasma supernatants were > 95% and 85% TCA-precipitable, respectively. Finally, selected blood samples were centrifuged through percoll gradients (which only allowed pelleting of the E). This procedure verified that cell-associated counts were only due to E binding. The same basic methodology was followed in our experiments with 10 kg rhesus monkeys, except 0.25 mg of HP was injected.

Quantitative Analyses. The specific activities of all proteins were determined and used to calculate the number of molecules bound per E for both *in vivo* and *in vitro* experiments. In the case of double or triple label experiments (^{125}I and ^{131}I, or both iodine labels and ^{51}Cr) the overlap of different labels was determined in calibration experiments and corrected counts were calculated for each isotope.

Assays for an Immune Response. We used a solid phase "Ag capture" assay, Khazaeli et al, J. Biol Resp. Med., 91:178 (1990) to determine if the animals developed an immune response to the injected agents (biotinylated mouse IgG, SA, and DNP_5BGG). This assay protocol

involves adhering the Ag to a solid matrix, adding a 10-20 fold dilution of the serum to be tested, and then adding radiolabelled Ag as a probe. Specific binding of antibody to solid phase Ag then allows capture of solution phase radiolabelled Ag. Positive controls in this assay included goat anti-mouse IgG, biotinylated human IgG, and mouse anti-DNP Mab (23D1), respectively. Alternatively, in order to increase the sensitivity of the assay, we "developed" with ^{125}I-Mab HB43, an anti-human IgG Mab which cross reacts with monkey IgG. This "direct" solid phase RIA, is considerably more sensitive for detecting a weak immune response (see below).

Results

In vitro preliminary studies in squirrel monkeys. We focused on the use of anti-CR1 Mab 1B4 in these clearance studies because it is known to block the "active site" (the C3b-binding site) of human CR1 (30) and therefore should bind close to the most "biologically relevant" site in terms of in vivo recognition by fixed receptors on liver or spleen cells. Also, large amounts of this Mab can be prepared from the hybridoma cell line. However, even though there are a moderate number of CR1 epitopes on the squirrel monkeys' E, defined by Mab E11, there are far fewer binding sites for Mab 1B4 on the monkey E compared to the number found on human E (Table 3A). Binding of Mab 1B4 to squirrel monkey E also appears to be of lower avidity than binding to human E, as evidenced by the relatively large reduction in net binding to the monkey E when the E are processed through a wash step, rather than simply by centrifugation through oil (Table 3A).

Construction of soluble, cross-linked multivalent A/B HP with biotinylated Mab $1B4_{10}$ considerably enhanced binding of this Mab to squirrel monkey E and caused an increase in binding to human E as well (Table 3B). Use of a radiolabel on the "second" (anti-target Ag) biotinylated Mab $23D1_5$ of the HP confirms that the A/B system does fix both biotinylated Mab to the primate E (Table 3B). The specificity of binding is confirmed by several experiments: "Background" binding of the HP to sheep E (lacking CR1) is much lower; and, in the presence of excess monomeric Mab 1B4 (in ascites), HP-mediated binding to human and squirrel monkey E is almost completely eliminated (Table 3C). The results also indicate that more HP-associated, biotinylated $23D1_5$, Mab is bound to the E than the biotinylated $1B4_{10}$ Mab, which is probably a consequence of amplification due to the multivalent nature of the A/B system. Finally, binding of the biotinylated $23D1_5$ Mab to primate E is reduced to background levels (the level seen for sheep E) unless both biotinylated $1B4_{10}$ and SA are used for construction of the HP (data not shown).

We used A/B HP prepared with biotinylated preparations of Mab 23D1 and either Mab 1B4 or Mab E11, to facilitate binding of the target Ag DNP_5BGG to primate E (Table 4) in a "whole blood" experiment analogous to those we have reported using SPDP-HP. It can be seen that comparable binding is obtained for HP constructed with either Mab $E11_{10}$ or $1B4_{10}$. We chose DNP_5BGG (a protein with a low degree of dinitrophenylation) as binding substrate for the in vivo experiments because it has been reported that a high degree of protein dinitrophenylation leads to rapid clearance from the circulation, even in the absence of specific antibody. The affinity of Mab 23D1 for the DNP group is only ca.

$5x10^6$ L/M, which is consistent with the moderate degree of binding (20-40%) of the DNP_5BGG by E opsonized with a HP containing $23D1_5$. A similar level of target Ag binding is demonstrable <u>in vivo</u> (see below).

<u>In vitro preliminary studies in cynomolgus and rhesus monkeys</u>. The level of detectable E11 and 1B4 epitopes on the E of these primates was higher than that detected for the squirrel monkeys (Table 5). In these experiments we prepared A/B HP using a "second" Mab, HB43, specific for human IgG. <u>In vitro</u> binding of these HP to monkey E is quantitated in Table 5.

<u>In vivo clearance kinetics in squirrel monkeys</u>. Injection of ^{125}I-DNP_5BGG into the squirrel monkey leads to rapid clearance of a fraction of the injected Ag counts within the first 15 min, followed by a slower phase of clearance (Figure 3). After 20 min we injected a $1B4_{10}/SA/23D1_5$ HP specific for both monkey CR1 and the DNP group. Within 10 min ca. 20% of the circulating Ag counts became E bound. Consistent with the uptake of counts by the E is a comparable loss of counts from the plasma. It is also evident that the majority of E-associated counts are then cleared rapidly from the circulation because a significant number of these counts do not return to the plasma phase. Finally, those plasma counts that did not become E-bound continued to be cleared at a relatively slow rate.

Clearance of both target Ag and HP were then followed through a double label experiment. The data in Figure 4A again demonstrates a rapid initial phase of Ag clearance followed by an approximate plateau and subsequent slow removal of plasma counts from the circulation. It should also be noted that less than 3%

of the counts are E-bound before the HP is injected (the first 25 min of the experiment). Upon injection of HP there is a drop in Ag-associated plasma counts which is ca. equal to the generation of E-associated counts (a maximum of 31% of the Ag was bound to the E). It is also evident that the majority of injected HP binds rapidly to the E (a maximum of 60%), and that subsequently both E-bound HP and Ag are cleared at approximately the same rate. The co-clearance of E-bound HP and Ag is also illustrated in a comparable experiment performed on a different squirrel monkey (Figure 4B). We also conducted an independent double label experiment similar to that depicted in Figure 4A, except the ^{125}I label was on the $23D1_5$ Mab (Figure 5A). The rate of clearance of E-bound HP and Ag was again approximately the same, and although maximum binding of Ag was 35% (comparable to earlier experiments), the maximum level of HP binding (defined by the ^{125}I label on the anti-DNP $23D1_5$ Mab) was reduced to 40%.

In order to address the potential problem associated with HP-mediated lysis of the E we co-injected ^{51}Cr labelled E along with ^{131}I-labelled DNP_5BGG, and followed both labels. After 35 min ^{125}I-labelled HP was injected and the clearance of all 3 radiolabels was monitored. Clearance of the ^{131} I- labelled DNP_5BGG (ca. 25% maximum E binding) followed the same trends seen in previous experiments (data not shown). Throughout the course of the experiment the ^{51}Cr label remained associated with the E pellet, and showed no significant change before or after injection of the HP (Figure 5B). In this experiment, 50% of the ^{125}I-labelled HP bound to the E, and the E-associated counts were cleared rapidly. Also, in agreement with the results shown in Figure 2, the fraction of HP that remained in the plasma was cleared at

a much slower rate.

<u>In vivo clearance kinetics in rhesus monkeys</u>. The level of HP binding (per E) to the squirrel monkey E was much higher than the level of Ag binding, and it is unlikely the Ag itself facilitated clearance of the majority of E-bound HP. However, it is important to demonstrate that the present results can be generalized to other primates and other Ag systems. For these reasons we used the A/B system to prepare HP with biotinylated Mab $1B4_{10}$ and human IgG_5, or with biotinylated Mab $E11_{10}$ and $HB43_5$ (an anti-human IgG Mab), and injected these HP into rhesus monkeys (Figures 6A and 6B). In these experiments we followed the HP with a radiolabel on the "second" antibody, rather than on the anti-CR1 Mab. The results clearly indicate that both these HP bind to the primate E and are then cleared rapidly. Once again the fraction of HP that did not bind to the E and remained in the plasma was cleared at a much slower rate. Consecutive experiments were performed on the same animals by injecting a second HP one hr after the first HP (Figure 6C). The overall clearance of the second HP from the animal's circulation was similar to that seen for the first injected HP.

We measured CR1 on E of the rhesus monkeys from blood aliquots obtained before and during these experiments, and the level remained constant (approximately equal to the levels reported in Table 5) during the course of the procedure. Finally, we tested these HP for complement activation and C3b capture after binding to human E in homologous serum. In some instances there was a low level of C3b binding to the E, but in other cases (e.g., the 1B4/human IgG HP) we could not detect any C3b bound to the E above background

levels.

In preliminary experiments (performed 3 weeks prior to those discussed above) we injected rhesus monkeys with a "partial HP" containing radiolabelled-biotinylated anti-CR1 Mab and SA only (lacking the "second" biotinylated antibody) in order to determine if this "partial" HP could bind E and be cleared from circulation. Although binding of this complex to E was approximately the same as that of a complete HP ($1B4_{10}/SA/23D1_5$), no significant short term clearance was observed, but after 3 weeks all counts had been cleared from circulation.

Development of an immune response. We conducted a total of 3 similar experiments on each of 2 squirrel monkeys over a period of 6 weeks. In order to determine if the squirrel monkeys developed an immune response to any of the injected materials (biotinylated mouse Mab, SA, or DNP_5BGG) we performed solid phase "Ag capture" assays on serum samples taken from the animals before, during, and after the experiments. The results of this assay indicate that there was no demonstrable immune response in any of the squirrel monkeys over a period of 10 weeks. However, by assaying with the more sensitive "direct" solid phase RIA (see Materials and Methods), we found that squirrel monkey 417 (but not 899) exhibited a rather weak, but demonstrable immune response to mouse IgG (but not SA or DNP_5BGG). The immune response was detected after the first experiment and increased slightly with time. Dose response experiments using the "direct" RIA indicate the binding capacity of a 5-fold dilution of serum from squirrel monkey 417 was comparable to that of a 10,000 fold dilution of goat antimouse IgG.

Two clearance experiments were performed on the rhesus monkeys, with a 3 week interval between experiments (see above). The "Ag capture" assay revealed no immune response to biotinviated mouse IgG during this time period. However, there was weak but measurable binding of mouse IgG by sera from both rhesus monkeys 2 weeks after the second experiment (data not shown). Using the more sensitive "direct" solid phase RIA (probing with ^{125}I-HB43) indicated the rhesus monkeys had developed a weak but demonstrable immune response to biotinylated mouse IgG by the start of the second clearance experiment. The titer of anti-mouse antibodies peaked 2 weeks after the second experiment and persisted in the circulation for more than 2 months. At the peak level (day 36), dose response experiments indicated that binding of the ^{125}I-HB43 probe corresponded to a titer of approximately 0.5% of that of a positive control, goat anti-mouse IgG (Table 6).

Table 3. Binding of anti-CR1 Mab and HP to Squirrel Monkey and Human E.

A. Direct CR1 Measurements[a]

mAb	Binding Assay	Molecules Bound per E[b]: S. Monkey 899	S. Monkey 417	S. Monkey 413	Human 1	Human 2
E11	Oil	310	280	290	578	510
E11	Washed Pellet	210	210	230	480	410
1B4	Oil	50	30	50	340	330
1B4	Washed Pellet	10	5	20	280	260
$1B4_{10}$[c]	Oil	55	30	60	310	310
$1B4_{10}$	Washed Pellet	10	4	20	240	230

B. "Whole Blood" Solution Phase Binding of HP[a,d]

HP	Molecules Bound per E[b]: S. Monkey 899	S. Monkey 417	S. Monkey 413	Human 1	Human 2
$^{125}I\text{-}1B4_{10}/SA/23D1_5$	640	470	600	1090	780
$1B4_{10}/SA/^{125}I\text{-}23D1_5$	4700	2330	5220	7460	4660

C. Inhibition of "Whole Blood" Solution Phase Binding by 1B4 Ascites

HP	Inhibitor	Molecules Bound per E[a,e]: S. Monkey 417	S. Monkey 413	Human 1	Human
$^{125}I\text{-}1B4_{10}/SA/23D1_5$	----	430	350	510	1000
$^{125}I\text{-}1B4_{10}/SA23D1_5$	1B4 Ascites[f]	54	43	60	-5

Footnotes for Table 3

a) The uncertainties in the reported values average ± 5%. In all cases controls with sheet E (which lack CR1) were used to subtract the background baseline level due to nonspecific binding.

b) E were separated from excess iodinated Mab by either spinning through an oil cushion or by pelleting and washing the E.

c) The subscripts refer to the molar input of biotin to Mab in the biotinylation reaction.

d) HP were assembled in solution and added to a dispersion of E in serum containing 0.01M EDTA, and bound molecules (Mab) were determined by counting the washed E pellets.

e) Half as much HP was incubated per E in part C compared to part B.

f) 1B4 ascites fluid was pre-incubated with the E in part C. Irrelevant ascites from an anti-C3b Mab gave the same results as the sample lacking inhibitor.

Table 4. HP-mediated Binding ("Whole Blood" Solution Phase Assay) of ^{125}I-DNP_5BGG to Squirrel Monkey and Human E.[a]

	% Bound				
HP	S. Monkey 899	S. Monkey 417	S. Monkey 413	Human	Sheep
$E11_{10}/SA/23D1_5$	28	38	20	34	1
$1B4_{10}/SA/23D1_5$	42	44	14	38	1
(Naive E)	2	2	2	1	1

Molar impact of $^{125}DNP_5$BGG corresponded to 37 molecules per E.

Table 5. Binding of Anti-CR1 Mab and HP to Rhesus Monkey and Cynomolgus Monkey E.

	Molecules Bound Per E[a]		
Mab[b]	Rh Monkey 4F	Rh. Monkey 941	Cy Monkey 69
E11	1760	1580	1480
1B4	230	140	110
HP[c]			
$1B4_{10}/SA/^{125}I\text{-}HB43_5$	710	700	480
$E11_{10}/SA/^{125}I\text{-}HB43_5$	620	610	460

a) See footnote a, Table 3.

b) Binding was determined by centrifugation through oil.

c) A relatively low input of HP per E was used. Binding values in a comparable experiment for an E Sample from a human were 1330 and 810 molecules per E for the HP containing $1B4_{10}$ and $E11_{10}$, respectively.

Table 6. Representative "Direct" Solid Phase RIA for Detecting an Immune Response to Biotinylated Mouse IgG in Rhesus Monkey 941.

A. Binding as a Function of Time[a]

Serum Source[b]	% ^{125}I-HB43 Bound to Solid Phase Matrix	
	$1B4_{20}$[c]	Mouse IgG[c]
pre-immune serum	0.8	0.8
Day 21	6.9	6.1
Day 36	9.5	15.3
Day 49	4.7	8.0
Day 80	1.9	2.9
goat anti-mouse IgG (Control)	23.2	22.2

B. Relative Titer (Day 36)

Serum and Dilution	% ^{125}I-HB43 Bound to Solid Phase Matrix[d]	
	$1B4_{20}$[c]	Mouse IgG[c]
Serum/20	12.2	15.6
Serum/80	4.0	4.2
goat anti-mouse IgG/200	36.5	37.0
goat anti-mouse IgG/1000	21.5	30.0
goat anti-mouse IgG/5000	4.6	9.4

a) The first injection (of the "partial" HP) was on day 1, and on day 21 the "complete" HP was injected.

b) All sera were examined at a 10 fold dilution.

c) Either $1B4_{20}$ or mouse IgG was used to first coat the plates (see Materials and Methods).

d) A different input of ^{125}I-HB43 was used in Part B compared to that in Part A.

METHODS OF USE

The franked RBC's described above have immediate application in a variety of research, clinical diagnostic, or therapeutic uses. The most important are therapeutic uses, which can include (1) using a franked RBC of the invention with specificity to an antigen such as HIV to clear free antigen from the blood of a human or primate patient, (2) using a franked erythrocyte with a specific Mab to clearfor a non-immunogenic but potentially "pathogenic" target such as LDL which has been linked to atherosclerosis and (3) using a franked erythrocyte with Mab specificity for the natural ligand of CR1 (such as C3b) where the number of naturally occurring receptors in an individual patient has decreased, such as in systemic lupus erythematosus. Of course, the specific antigens or antibody targets identified above are exemplary only, and virtually any circulating microorganism, virus, compound and the like to which a Mab can be prepared can be subject to therapeutic treatment through the invention.

The franked erythrocytes may be prepared and introduced for therapeutic use in either of three methods. First, the bi-specific heteropolymer comprised of at least two cross-linked Mab, one specific for the RBC and the other for the antigen, may be introduced directly to the bloodstream through inoculation. Alternatively, a small amount of RBC's can be extracted from the patient, and bound to the heteropolymer in sterile <u>in vitro</u> conditions and then reintroduced into the patient. Finally, in cases of low CR1 or low RBC disease states, franked erythrocytes from a compatable heterologous matched blood donor can be used. In any of the above examples, a "cocktail" of several

heteropolymers (see results section) can be used. Given the high binding capacity of the heteropolymer to the RBC, direct injection of the heteropolymer can be as effective as in vitro preparation of the franked erythrocyte, followed by inoculation.

In addition to taking advantage of the body's natural defenses by augmenting the natural immune defense system, binding the heteropolymer to the RBC's (either in vitro or in vivo) may reduce or remove any "immunogenicity" that would be characteristic of Mabs prepared from mouse hosts and the like. In fact, currently, the vast majority of monoclonal antibodies are produced in mice. Mab treatment thus suffers, at least to some degree, from the body's natural immune response against the mouse Mab which would thus prevent the Mab from binding to its target antigen. As the number of heteropolymers bound per RBC is relatively small (below about 500) the Mab itself may not be recognized as foreign, and the host immune response may not be triggered, or at least, will be significantly reduced. This appears to be born out by the results reported. Use of available human Mabs to prepare heteropolymers should also eliminate any host immune response.

The dosage and treatment regimen will vary from antigen to antigen, individual to individual, and disease state. In general, these can be determined on an empirical basis. An extreme minority of available RBC's may be used effectively in conferring therapeutic treatment. This is due in part to the vast numbers of RBC's present in the blood, in contrast to most antigens. As an example, the level of HIV which circulates free in the blood has been suggested as the most cytopathic form of HIV. High levels of HIV in the circulation appear to

correlate with disease activity. Yet, this level ranges between 1,000 and 50,000 virus particles per ml. Ho et al, New England Journal of Medicine, 321, 1621-1625 (1989), Coombs et al., ibid, 1626-1631. In contrast, the number of RBC per ml in circulation is many orders of magnitude greater, and accordingly, even a small minority of available RBC's treated according to the claimed invention should be sufficient to confer therapeutic treatment, given the appropriate anti-CR1/anti-HIV franked RBC. As exemplary levels only, in the treatment of AIDS, an intravenous administration of no more than 1-4 mg of appropriate heteropolymer should be sufficient to frank the patients' RBC's for quantitative binding of circulating HIV. With sufficiently high avidity anti-HIV antibodies in the heteropolymer (easily achieved by standard methods) it should be possible to use considerably less heteropolymer (μg amounts). See the " Detailed Calculations Section" for a more complete analysis of this problem. Alternatively, if the RBC's of the patient are first removed and franked with heteropolymer and then re-injected, the dose administration of franked erythrocytes would be considerably less than 1 "unit" (1 pint) of blood. Use of ca. 50-100 ml of franked RBC's (a few % of total circulating RBC's) should be adequate. The low levels needed are a consequence of the fact that even under conditions of high disease activity the concentration of infectious agent in the blood (e.g. HIV) is many orders of magnitude lower than the concentration of RBC's.

This invention has been described by reference both to generic description and specific embodiment. Examples provided are not intended to be limiting unless so specified, and variations will occur to those of ordinary skill in the art without the exercise of inventive

faculty. The invention embraces these alternatives, save for the limitations imposed by the claims set forth below.

Claims

1. A mammalian erythrocyte bound to a first monoclonal antibody at a receptor site for which said first monoclonal antibody is specific, said first monoclonal antibody being cross-linked to a second monoclonal antibody specific for an antigen present in the mammalian primate circulatory system.

2. The erythrocyte of Claim 1, wherein said erythrocyte bears, on its surface, up to 1000 of said first monoclonal antibodies bound to said second monoclonal antibody.

3. The erythrocyte of Claim 1, wherein said erythrocyte receptor site is the CR1 protein.

4. The erythrocyte of Claim 1, wherein said antigen is selected from the group consisting of a virus, microorganism or toxic chemical.

5. The erythrocyte of Claim 1, wherein said antigen is a non-immunogenic substance found in the mammalian primate circulatory system, which can become pathogenic or cause adverse biological effects if present in sufficient amounts.

6. The erythrocyte of Claim 4, wherein said antigen is HIV (The AIDS virus).

7. The erythrocyte of Claim 5, wherein said antigen is low density lipoprotein.

8. The erythrocyte of Claim 1, wherein said erythrocyte is a human or other primate erythrocyte.

9. The erythrocyte of Claim 8, wherein said monoclonal antibodies are obtained from a non-human host, or from human sources.

10. A method of therapy treating a mammalian primate individual having an antigen present in its circulatory system, comprising inoculating said individual with a monoclonal antibody heteropolymer comprising a first monoclonal antibody specific for a receptor site on the surface of the erythrocyte of said individual, said first monoclonal antibody being cross-linked to a second monoclonal antibody specific for said antigen, said inoculation being performed in sufficient amounts to permit binding of sufficient antigen to erythrocytes via said antibodies to reduce the amount of free antigen in circulation in said individual to a level below that which is cytopathic or which causes adverse biological effects to said individual. The inoculation may be performed either directly with said heteropolymer, or indirectly by franking the patient's erythrocytes (or erythrocytes from a compatible donor) with said heteropolymer, followed by inoculation of the franked erythrocytes.

FIG. 1a

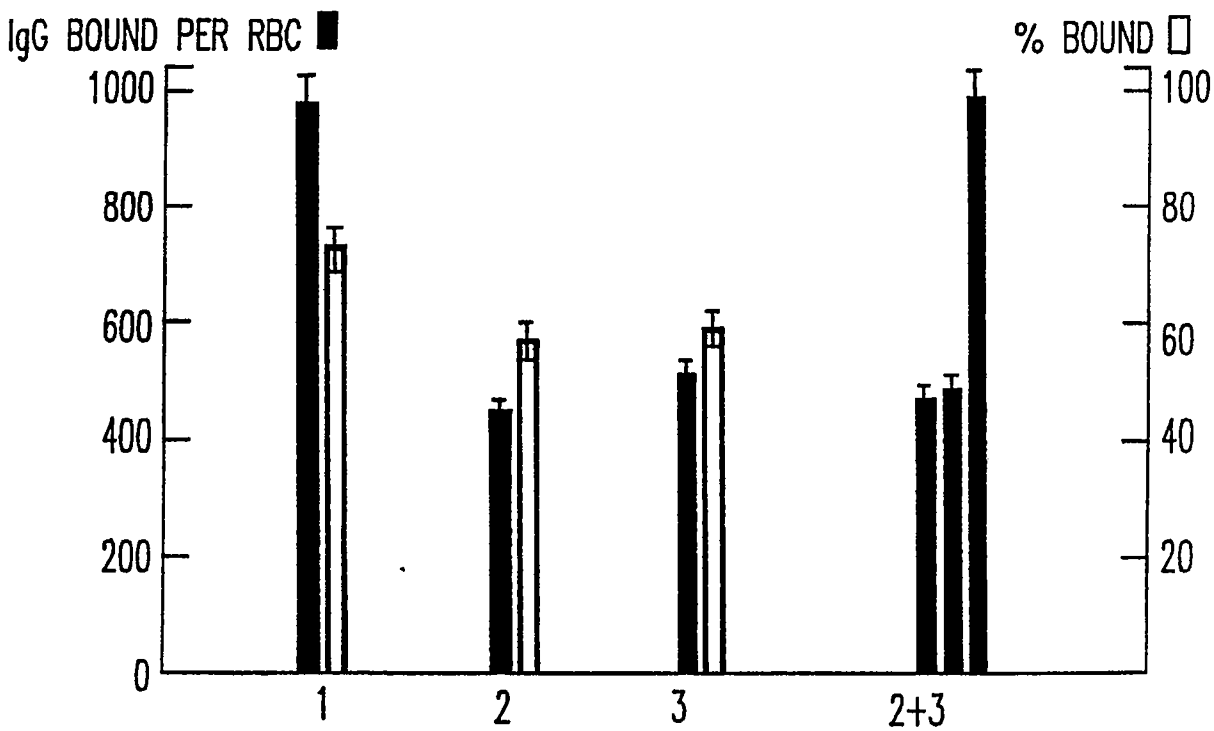
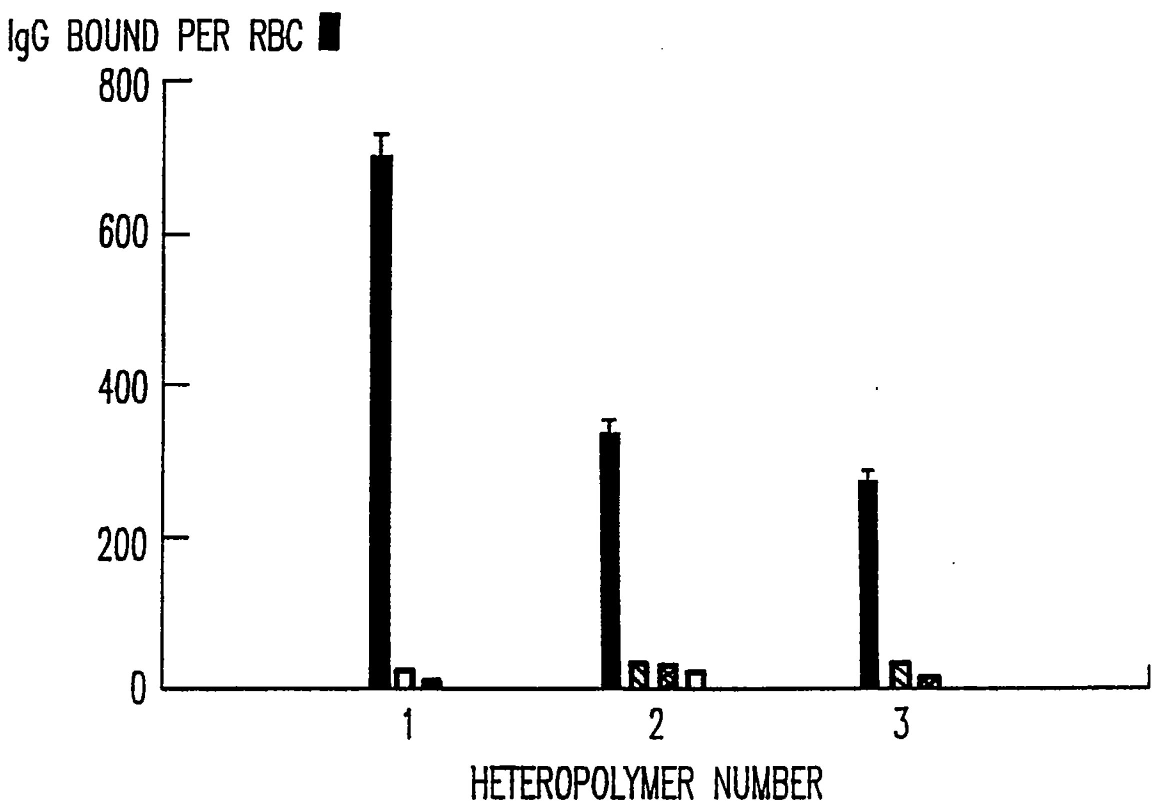

IgG BOUND PER RBC
% BOUND
1000
800
600
400
200
0
100
80
60
40
20
1
2
3
2+3

FIG. 1b


IgG BOUND PER RBC
800
600
400
200
0
1
2
3
HETEROPOLYMER NUMBER

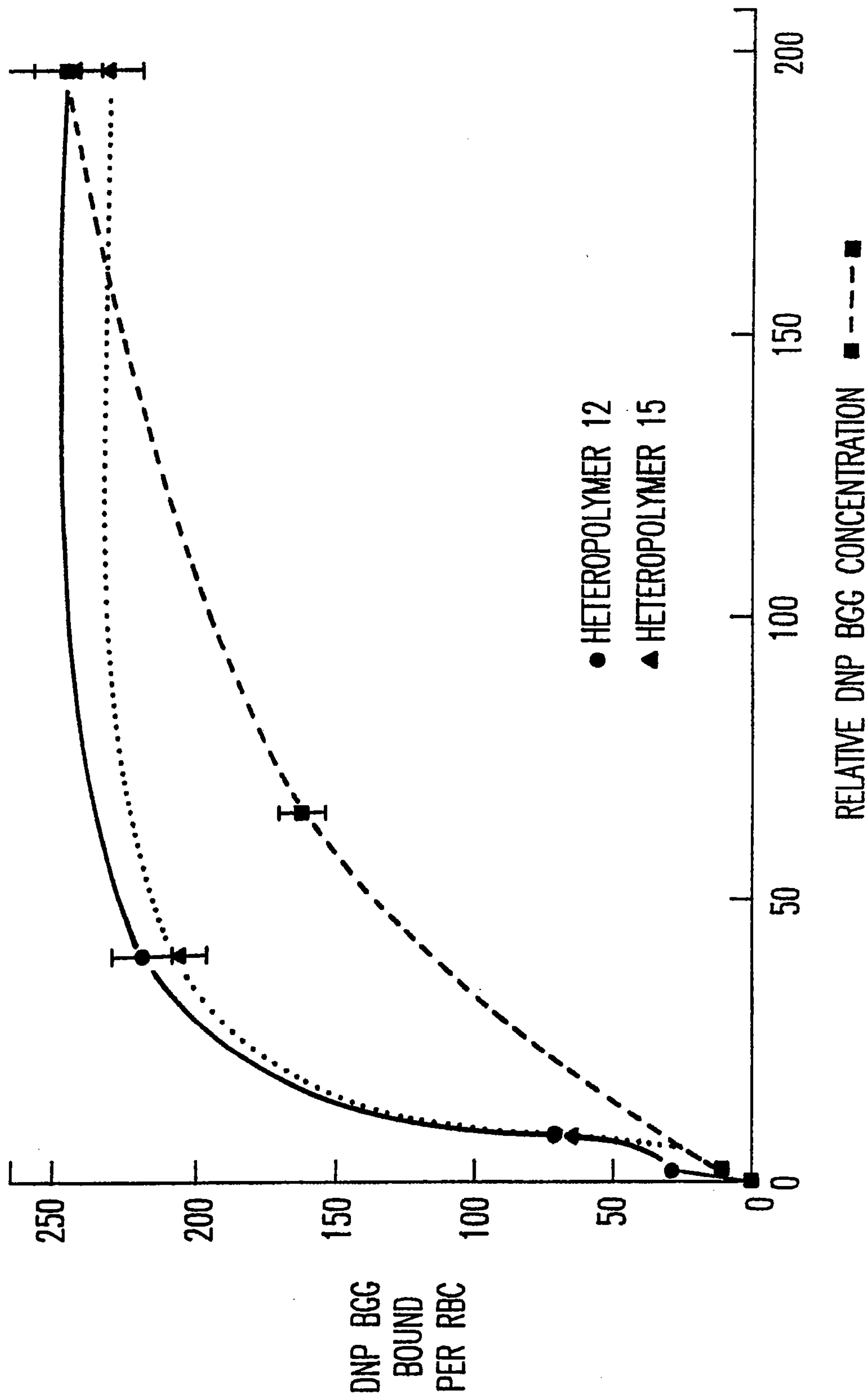
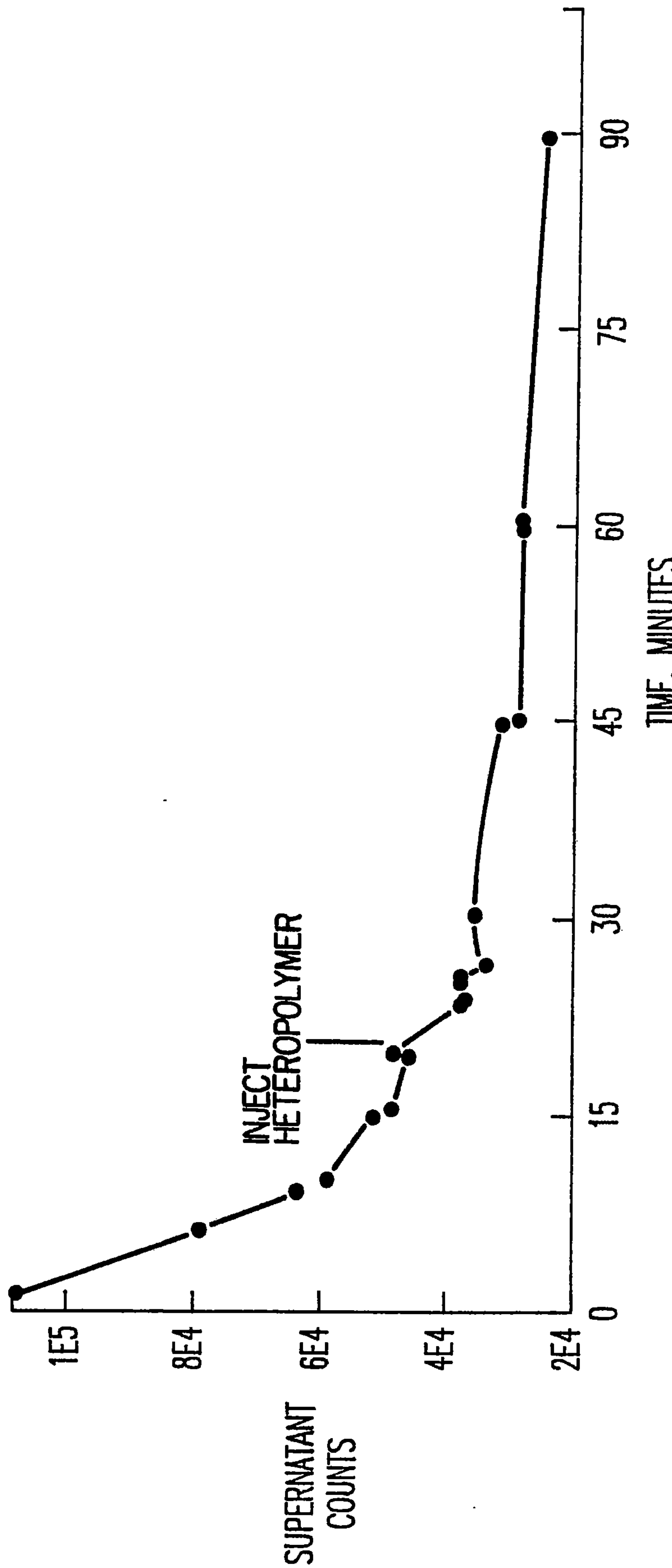

DNP BGG
BOUND
PER RBC
0
50
100
150
200
250
0
50
100
150
200
RELATIVE DNP BGG CONCENTRATION
● HETEROPOLYMER 12
▲ HETEROPOLYMER 15
■ - - - ■

FIG. 2



SUPERNATANT COUNTS
1E5
8E4
6E4
4E4
2E4
0
15
30
45
60
75
90
TIME, MINUTES
INJECT HETEROPOLYMER

FIG. 3a

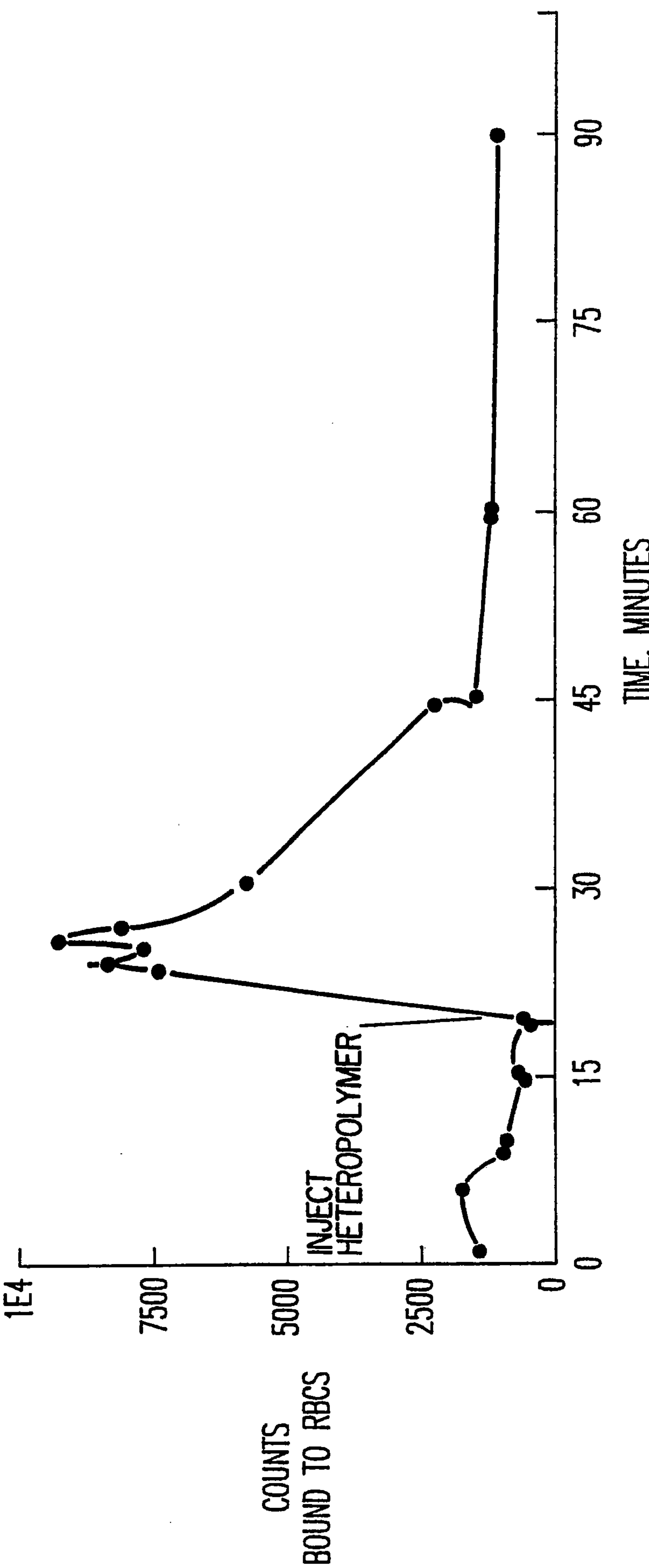
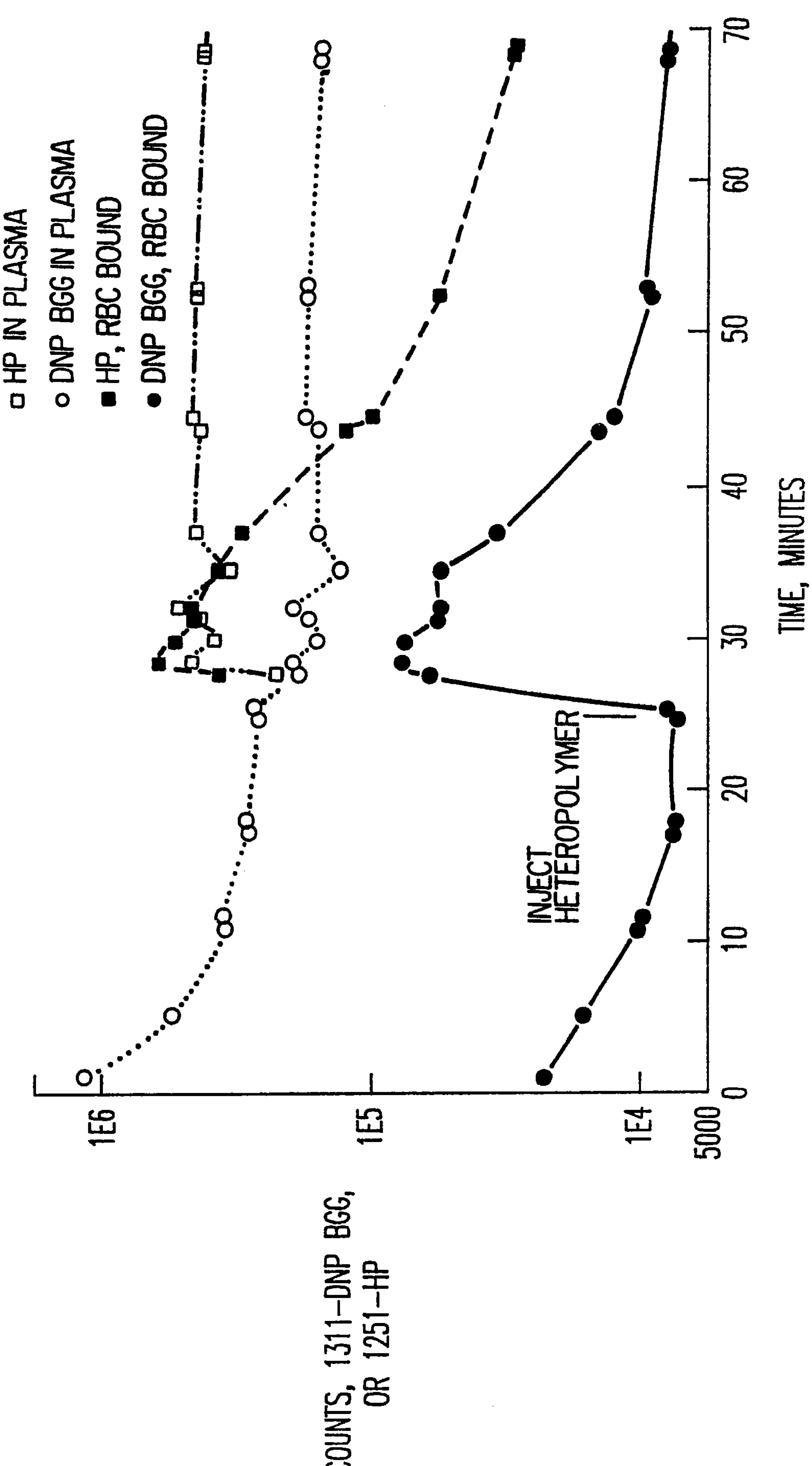

COUNTS
BOUND TO RBCS
1E4
7500
5000
2500
0
INJECT
HETEROPOLYMER
0
15
30
45
60
75
90
TIME, MINUTES

FIG. 3b



□ HP IN PLASMA
○ DNP BGG IN PLASMA
■ HP, RBC BOUND
● DNP BGG, RBC BOUND
INJECT HETEROPOLYMER
TIME, MINUTES
0
10
20
30
40
50
60
70
1E6
1E5
1E4
5000
COUNTS, 131I—DNP BGG, OR 125I—HP

FIG. 4a

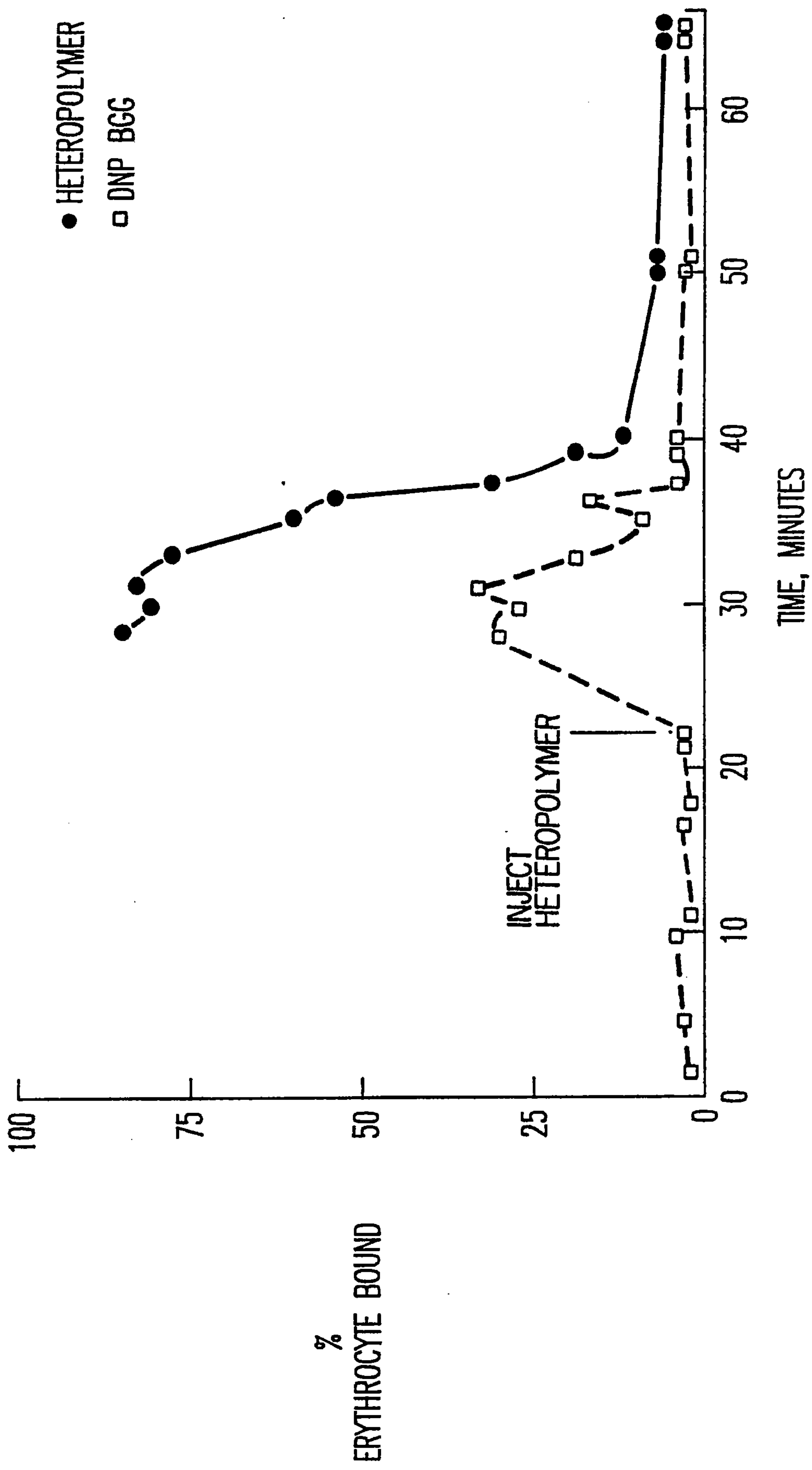

● HETEROPOLYMER
□ DNP BGG
% ERYTHROCYTE BOUND
100
75
50
25
0
0
10
20
30
40
50
60
TIME, MINUTES
INJECT HETEROPOLYMER

FIG. 4b

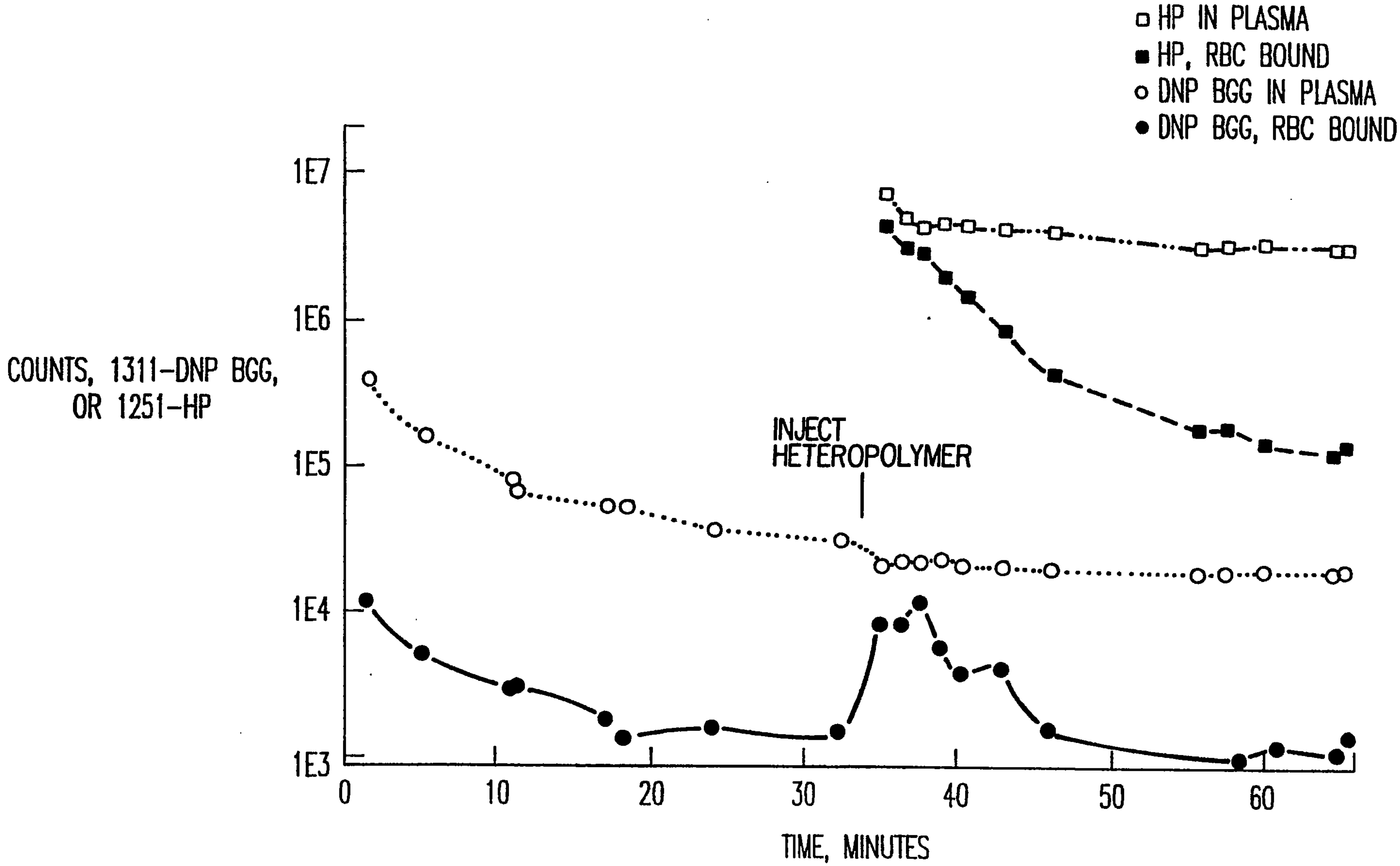

HP IN PLASMA
HP, RBC BOUND
DNP BGG IN PLASMA
DNP BGG, RBC BOUND
COUNTS, 131I-DNP BGG, OR 125I-HP
1E7
1E6
1E5
1E4
1E3
INJECT HETEROPOLYMER
0
10
20
30
40
50
60
TIME, MINUTES

FIG. 5a

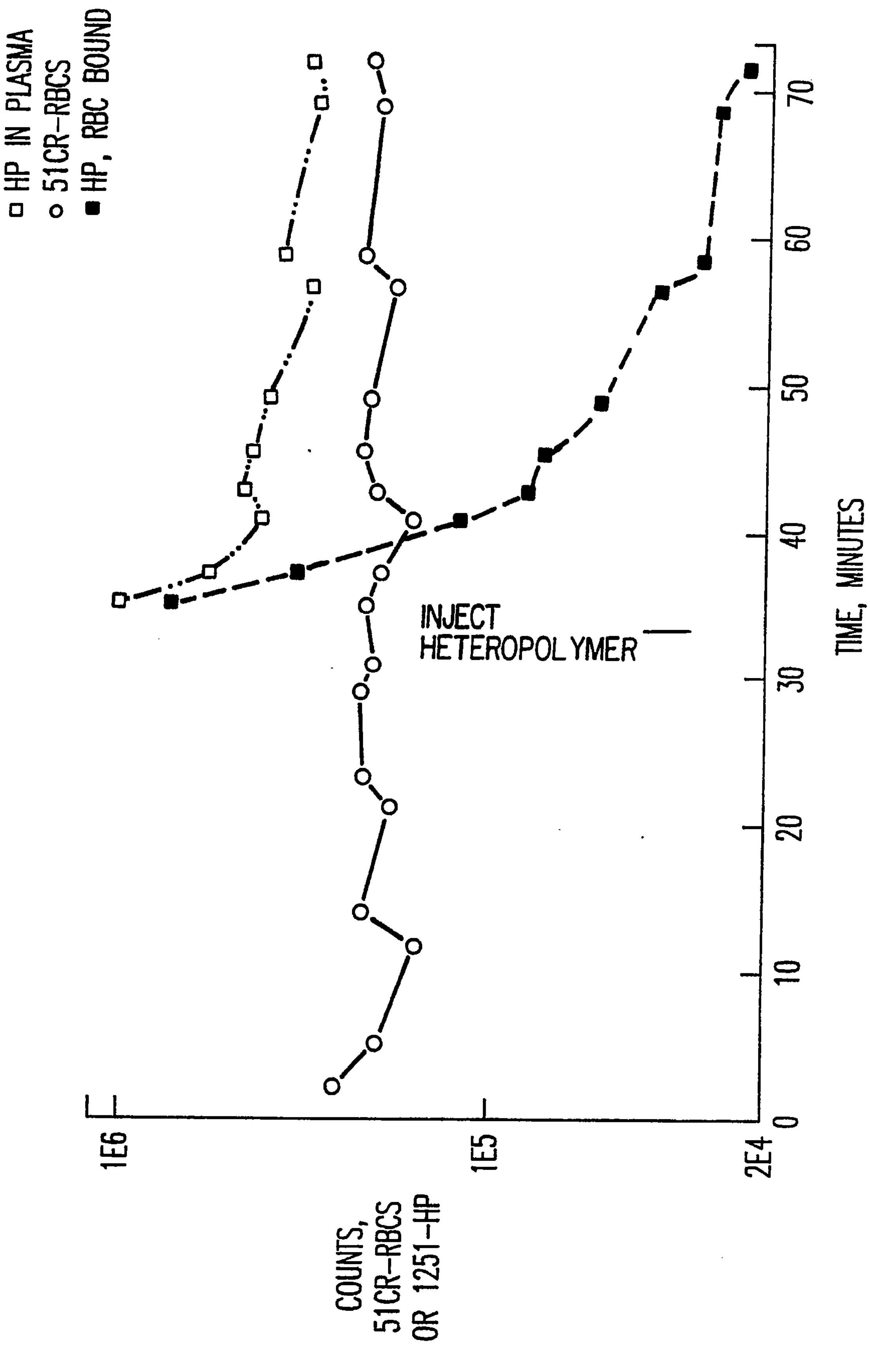

HP IN PLASMA
51CR-RBCS
HP, RBC BOUND
INJECT HETEROPOLYMER
COUNTS, 51CR-RBCS OR 1251-HP
1E6
1E5
2E4
0
10
20
30
40
50
60
70
TIME, MINUTES

FIG. 5b

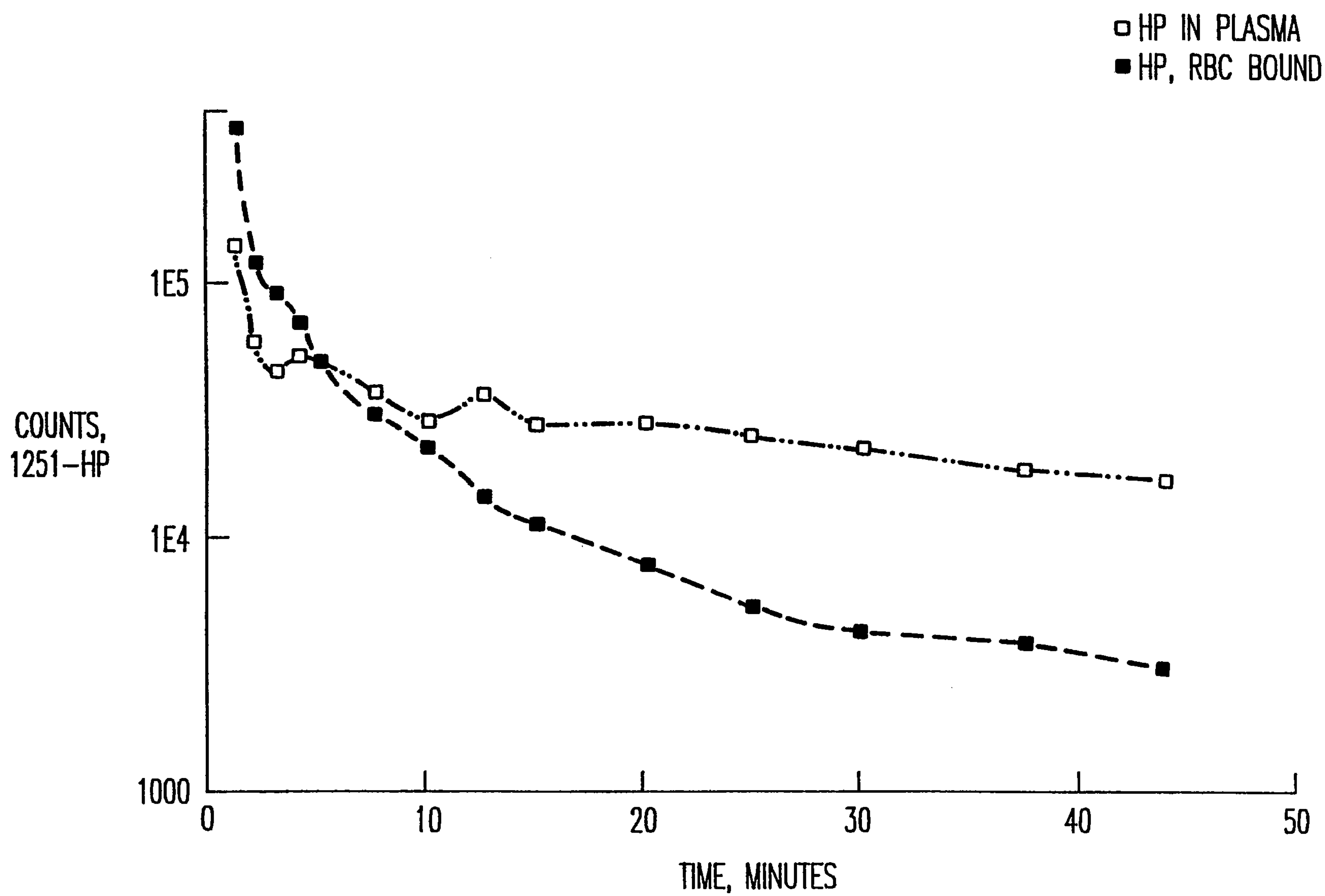

HP IN PLASMA
HP, RBC BOUND
COUNTS,
1251-HP
1E5
1E4
1000
0
10
20
30
40
50
TIME, MINUTES

FIG. 6a

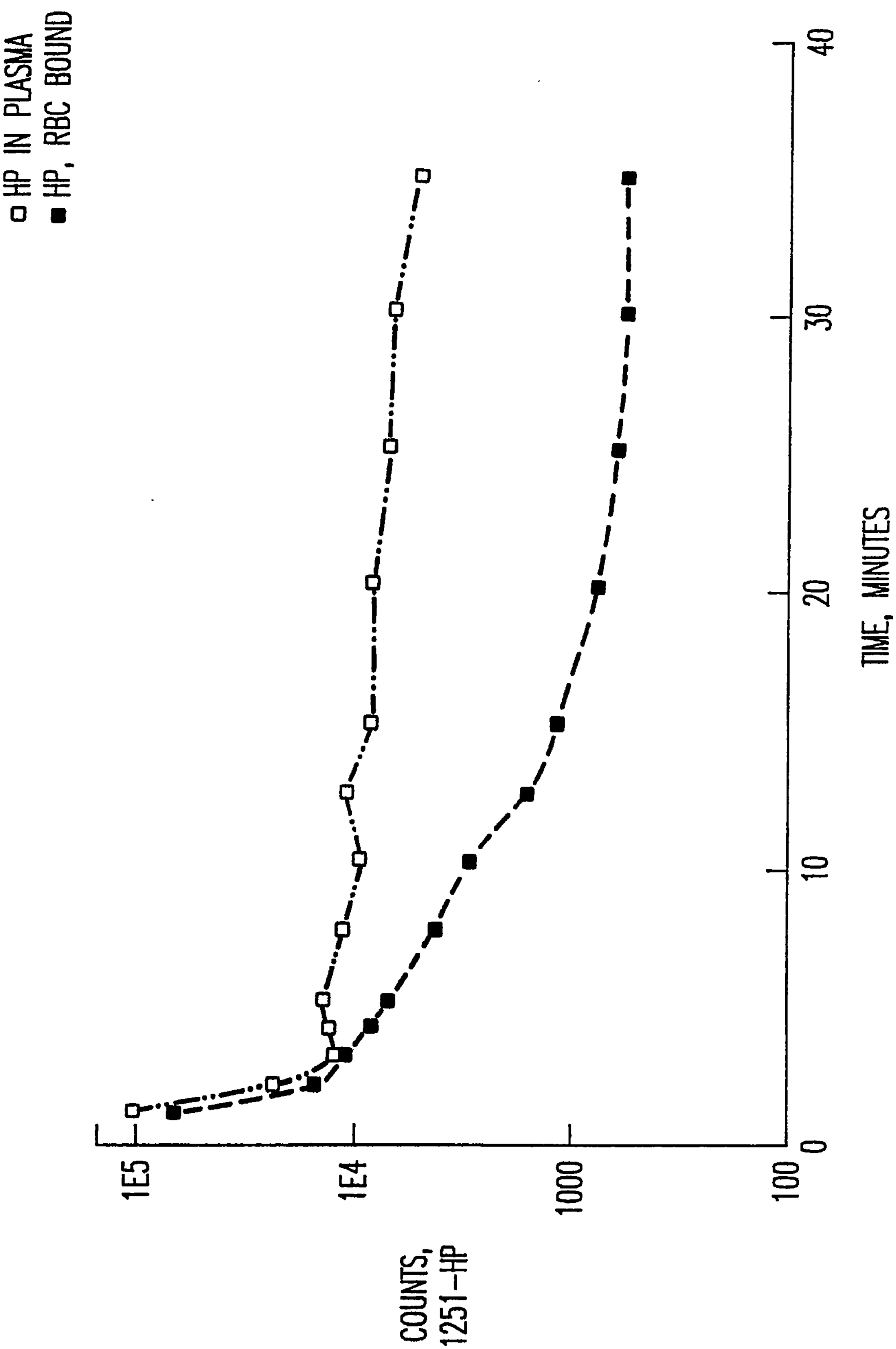

HP IN PLASMA
HP, RBC BOUND
COUNTS,
1251–HP
1E5
1E4
1000
100
0
10
20
30
40
TIME, MINUTES

FIG. 6b

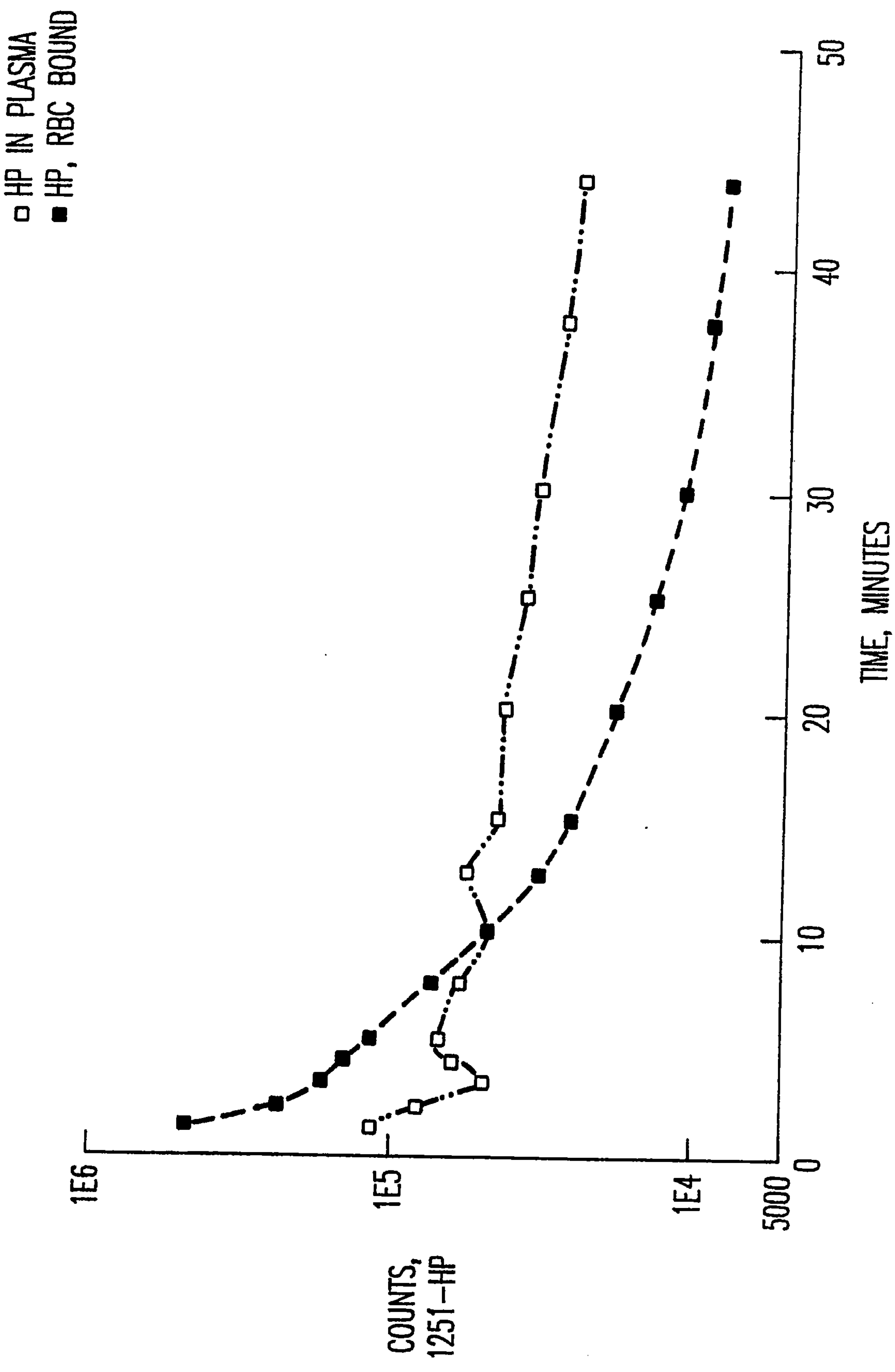

HP IN PLASMA
HP, RBC BOUND
COUNTS,
1251-HP
1E6
1E5
1E4
5000
0
10
20
30
40
50
TIME, MINUTES

FIG. 6c

INTERNATIONAL SEARCH REPORT

International Application No PCT/US91/07158

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): A61K 39/395 , 35/14

U.S. CL.: 435/2; 424/85.8; 604/4

II FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
U.S.	435/2; 424/85.8; 604/4

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	Journal of Experimental Medicine, Volume 160, issued December 1984, Karpovsky et al., "Production of Target-Specific Effector Cells Using Hetero-Cross-Linked Aggregates Containing Anti-Target Cell and Anti-Fc Receptor Antibodies", pages 1686, lines 6-11 and 21-22: page 1687, lines 1-10: page 1688, lines 4-22: page 1691, lines 1-10 and figure 2: page 1697, Table V and lines 14-29.	1-10
Y	The Journal of Immunology, Volume 139, Number 9, issued 01 November 1987, Titus et al. "Human K/Natural Killer Cells Targeted With Hetero-Cross-Linked Antibodies Specifically lyse Tumor Cells In Vitro and Prevent Tumor Growth In Vivo", pages 3153-3158. See Abstract.	1-10

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
16 January 1992	30 JAN 1992
International Searching Authority	**Signature of Authorized Officer**
ISA/US	George C. Elliott ebw

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	The Journal of Immunology, Volume 139, Number 11, issued 01 December 1987, Edberg et al. "Quantitative Analysis of The Binding of Soluble Complement-Fixing Antibody/dsDNA Immune Complexes to CR1 on Human Red Cross Cells", pages 3739-3747. See entire article.	1-10